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PATENT- OG VAREMÆRKESTYRELSEN

Methods for clonal derivation of human blastocyst-derived stem cell lines

FIELD OF THE INVENTION

The present invention relates to methods and optimized conditions for clonal derivation of a human blastocyst stem cell line, wherein the cell line can be propagated in an undifferentiated state while maintaining its pluripotency both *in vitro* and *in vivo*.

BACKGROUND OF THE INVENTION

Human blastocyst-derived stem cells (hBS) are undifferentiated pluripotent cells that can differentiate to a variety of specialized cells. Human BS stem cell lines have widespread implications for e.g. therapeutic treatment, human developmental biology, drug discovery and drug testing.

To date, while stem cell lines have been isolated from human blastocysts in various laboratories, only very few of the cell lines have been subcloned to obtain a homogenous cell line derived from one single cell, as clonal derivation of hBS stem cell lines are associated with many obstacles such as low survival rate and loss of clones to irreversible differentiation. In previous attempts very low success rates have been reported (<0.1%). The hBS cells are assumed to be sensitive to even small changes in the micro-environment and are conventionally cultured and passaged as colonies or parts thereof. Accordingly, the dissociation into single cells is one of the critical steps in the art of subcloning.

Furthermore, each stem cell line seem to require different conditions for clonal expansion, i.e. the conditions that works well with one stem cell line may be ineffective for the subcloning and maintenance in an undifferentiated stage of another stem cell line.

As a result there are only few cloned cell lines available. Since the originally described hBS cell line were not clonally derived from single cells but the inner cell mass of the blastocyst, the pluripotency has not often been demonstrated on the original cell line. Due to this, the formal possibility exist that within a population of homogeneous-appearing cells there are actually multiple precursor or stem cells committed to different lineages. This could mean in fact that no single cell is capable of forming derivatives of all three embryonic germ layers. To determine the pluripotency in human cell cultures, one has to be sure that the cells within a colony are descendants from

one single cell. Thus, the obtainment of a pure cell line is essential for the proper use of human blastocyst-derived stem cells and accordingly, there is a need for methods for obtaining cloned stem cell lines, i.e. stem cell lines derived from one single cell.

DESCRIPTION OF THE INVENTION

Accordingly, the present invention provides methods and optimized conditions for clonal derivation of a human blastocyst stem cell line, wherein the cell line can be propagated in an undifferentiated state while maintaining its pluripotency both *in vitro* and *in vivo*.

The invention also relates to clonal derivation of derivatives of hBS cells, e.g., neural, myocardial, hepatic and pancreatic progenitor cells.

Accordingly, one aspect of the invention relates to a method for clonal derivation of human blastocyst-derived stem cells (hBS) or derivatives thereof, the method comprising the steps of

a) dissociation of hBS cell colonies into one or more single hBS cells,

b) cultivation of the one or more single hBS cells,

to obtain one or more hBS cell clones which are derived from a single hBS cell and are capable of forming colonies.

The human blastocyst-derived stem cells or stem cell line to be cloned may be prepared by a method as described in PCT/EP02/14895 (1).

In order to obtain clones of hBS cells, the cultivation in step b) above may be performed in a medium that promotes propagation of the one or more hBS cells.

The present inventors have developed a medium suitable for clonal derivation of hBS cells, especially for hBS cells prepared by a method as described in PCT/EP02/14895. However, the medium may also be applicable for clonal derivation of other stem cell lines, either used in its present form or with suitable adjustments of the components and/or the amounts of the components.

The medium is denoted cell free hBS cell conditioned cloning medium (CC medium) and comprises a concentrated conditioned medium (CC-base), and, optionally, a suitable cultivation medium.

A conditioned medium according to the invention is prepared by culturing a population of cells in a medium, and then harvesting the medium.

In a specific method according to the invention the concentrated conditioned medium may be prepared by the following steps:

1) cultivating hBS cells in a serum based medium, such as FCS (Fetal Calf Serum) or human serum to obtain a conditioned medium,
2) collecting the conditioned medium within suitable time intervals,
3) concentrating the conditioned medium,

to obtain the concentrated conditioned medium (CC base).

Step 1) may be performed in the presence of feeder cells, such as, e.g. embryonic fibroblasts, or under feeder cell free conditions (see below for a more throughout discussion).

The serum based medium may comprise from about 60% v/v to about 90% v/v KO-DMEM (KnockOut Dulbecco's Modified Eagle Medium), from about 5% v/v to about 30% v/v FCS or human serum, from about 1 mM to about 10 mM glucose and from about 1 ng/ml to about 20 ng/ml bFGF (basic fibroblast growth factor).

The serum based medium may further comprise from about 0.1% to about 5% PEST (penicillin/streptomycin), from about 0.1% to about 5% v/v glutamin or a chemical equivalent, such as Glutamax and/or from about 0.1% v/v to about 5% v/v NEAA (non essential amino acids).

In a specific embodiment of the invention, the serum based medium is a FCS based medium, comprising 15% FCS, 3.5 mM glucose, 1% PEST, 1% Glutamax, 1% NEAA and 4 ng/ml bFGF in KO-DMEM.

The medium may be collected in step 2) at least every 12th hour, such as, e.g. at least every 18th hour, at least every 24th hour, at least every 36th hour, at least every 48th hour or at least every 60th hour.

Step 3) may be performed by concentrating the conditioned medium by a factor from about 2 to about 10, such as, e.g. from about 2 to about 9, from about 2 to about 8, from about 3 to about 7, from about 4 to about 6 or from about 4 to about 5 by use of a

suitable concentrating column.

In a specific embodiment of the invention, the conditioned medium is concentrated by a factor 4, e.g. from about 50 ml to about 12.5 ml, by use of a Centriprep concentration column WM50 spun at 1500 g, but any other column with a suitable cut-off and any other suitable centrifugation conditions may of course be used.

The cell free hBS cell conditioned cloning medium may comprise at least 5% v/v, such as, e.g., at least 7.5% v/v, at least 10% v/v, at least 12.5 % v/v, at least 15% v/v, at least 17.5% v/v, at least 20% v/v, at least 25% v/v, at least 30% v/v, at least 35% v/v, at least 40% v/v, at least 45 % v/v, at least 50% v/v, at least 55% v/v, at least 60% v/v, at least 65% v/v, at least 70% v/v, at least 75% v/v, at least 80% v/v, at least 85% v/v, at least 90% v/v, at least 95% v/v or 100% v/v of the concentrated conditioned medium (CC-base) and, optionally, a suitable cultivation medium.

In one aspect of the invention the cultivation medium in the cell free hBS cell conditioned cloning medium (CC medium) is KO-DMEM medium.

The cell free hBS cell conditioned cloning medium may further comprise at least one of the following: glucose, Glutamax, NEAA, PEST and/or a growth factor such as, e.g., bFGF, EGF (epithelial growth factor), HGF (hepatic growth factor) and/or FGF4.

In a specific method according to the invention the cell free hBS cell conditioned cloning medium may comprise 82% KO-DMEM (v/v), 15% concentrated conditioned medium (CC base), 3.5 mM D-glucose, 4 ng/ml bFGF, 1% v/v PEST, 1% v/v Glutamax and 1% v/v NEAA.

Contrary to the findings of other researchers, a medium comprising serum has shown to be favorable for the clonal derivation of hBS cells. The medium may be used in step b) in the method for clonal derivation as described above, and is denoted Fetal Calf Serum (FCS) based medium and comprises at least 5% v/v FCS, such as, e.g., at least 7.5% v/v FCS, at least 10% v/v FCS, at least 12.5 % v/v FCS, at least 15% v/v FCS, at least 17.5% v/v FCS, at least 20% v/v FCS, at least 25% v/v FCS, at least 30% v/v FCS, at least 35% v/v FCS, at least 40% v/v FCS or at least 45 % v/v FCS and a suitable cultivation medium.

The cultivation medium may be KO-DMEM medium, and may further comprise a growth factor, such as, e.g. bFGF, EGF, HGF and/or FGF4, and/or glucose.

More specific, the FCS based medium may comprise from about 60% v/v to about 90% v/v KO-DMEM medium, from about 5% v/v to about 30% v/v FCS, from about 1 mM to about 10 mM glucose and from about 1 ng/ml to about 20 ng/ml bFGF, and may further comprise Glutamax, NEAA and/or PEST.

Another suitable medium for use in step b) a method according to the invention is a human serum based medium comprising human serum and a suitable cultivation medium.

The cultivation of the one or more single hBS cell in step b) in a method as described above may be performed in the presence of feeder cells, i.e. cells of one type that are co-cultured with cells of another type, to provide an environment in which the cells of the second type can proliferate. The feeder cells may typically be inactivated when being co-cultured with other cells by irradiation or treatment with an anti-mitotic agent such as mitomycin c, to prevent them from outgrowing the cells they are supporting.

Examples of suitable feeder cells are embryonic fibroblasts, such as, e.g. mouse embryonic fibroblasts, human foreskin fibroblasts, fetal skin fibroblasts, fetal muscle fibroblasts, human adult skin fibroblasts and fibroblasts derived from hBS cells.

In order to minimize the risk of differentiation, 50% (v/v) of the medium used in step b) in a method as described above may be changed to a hBS cell culture medium comprising 77% KnockOut DMEM, 20% Serum Replacement, 1% Glutamax, 1% NEAA, 1% PEST and 4ng/ml bFGF. The first medium change is performed at least 24 hours after the start of the cultivation of the single hBS cells.

Cells are traditionally cultured on a layer of feeder cells in order to promote cell survival, proliferation and colony formation. Unfortunately, using feeder cells increases production costs, impairs scale-up, and produces mixed cell populations that require the clones to be separated from feeder cell components. Furthermore, for therapeutic applications it will be of greatest importance that the cells are cultured without xenogenic tissue contact, such as, e.g. feeder cells. For screening applications the cells need to be cultured in absence of other supporting cells, no matter the origin of

the supporting cells.

Accordingly, the invention also relates to a method wherein the cultivation of one or more hBS cells in step b) is performed under feeder cell free conditions.

The presence of a suitable medium, such as, e.g. a tissue culture medium, and a support medium, i.e. a growth support substrate or coating, is very important when growing cells under feeder free conditions. When cultivating hBS cells on feeder cells, the feeder cells excrete various substances that promote colony formation and proliferation and inhibit the differentiation of the hBS cells. When growing cells under feeder free conditions such substances have to be supplemented to the growth medium or coated on to the surfaces of the tissue culture wells. Certain surfaces or surface treatments comprising components for stimulating cell adhesion, colony formation or for inhibiting differentiation can be applied, such as e.g., extra cellular matrix components such as, e.g., Matrigel® or laminin, or other components, such as, e.g. albumin, gelatine, polyornithine, fibronectin, vitronectin, agarose, poly-L-lysine or different collagen subtypes, such as, e.g. collagen type I, and/or combinations thereof.

The dissociation of the hBS cell colonies in step a) in a method according to the invention may be done without the use of enzymatic treatment. Normally, cell colonies are dissociated by use of e.g. trypsin, but the present hBS cell lines form very tight complexes, and may not be fully dissociated by the use of enzymatic treatment alone. Furthermore, a milder treatment than enzymatic treatment is also desired in order to maintain the membrane structure of the hBS cells.

Accordingly, the invention relates to a method wherein the hBS cell colonies in step a) are dissociated by use of a mechanical method.

The mechanical method may comprise the steps of

i) cutting hBS cell colonies to obtain smaller units, having a size between about 200 μm x about 200 μm to whole colonies,

ii) incubating the smaller units with a medium containing a chelator such as, e.g., EDTA,

iii) triturating the smaller units to obtain hBS single cells..

The method may further comprise a step of dispersing the hBS single cells in a suitable

medium, such as, e.g. a cell free hBS cell conditioned cloning medium (CC-medium), FCS based medium or the hBS culture medium mentioned above.

The invention also relates to a method for preparing a concentrated conditioned medium (CC base), the method comprising

1) cultivating hBS cells in a serum-based medium, such as, e.g., FCS or human serum,
2) collecting the conditioned medium within suitable time intervals,
3) concentrating the conditioned medium,

to obtain the concentrated conditioned medium (CC base).

Furthermore, the invention relates to a hBS cell conditioned cloning medium comprising a concentrated conditioned medium prepared by a method as described herein.

Other applications

Other aspects of the invention appear from the appended claims. The details and particulars described above and relating to the method according to the invention apply *mutatis mutandis* to the other aspects of the invention.

FIGURE LEGENDS

Figure 1 is a schematic picture of the preparation of concentrated conditioned medium (CC base).

Figure 2 shows a colony derived from one single cell of cell line AS034 (later referred to as AS034.2)15 days after being seeded. The picture was taken before transfer from the 96-well-plate.

Figure 3 shows subclone AS034.2 after passage 8, 6 days.

Figure 4 is a diagram showing the effects of three different media supplements on colony forming from 1000 and 100 cells seeded per well respectively. Five wells in two separate plates were used for each combination of media supplement and cell concentration. The experiment was performed twice and four times respectively for two different cell lines.

Figure 5 shows AS034.1 - staining positive for the undifferentiated marker SSEA-4.

Figure 6 shows AS034.1 cultured 12 days and staining positive for the endodermal marker HNF3β (*in vitro* differentiation).

Figure 7 shows neuroectodermal tissue from teratoma formation of AS034.1.

Figure 8 shows secretory epithelium (endoderm) from teratoma formation of AS034.1.
Figure 9 shows early cartilage (mesoderm) from teratoma formation of AS034.1.

The following examples tend to illustrate the invention without limiting it hereto.

EXAMPLES

Materials and methods

Example 1
Establishment of hBS cell line from spontaneously hatched blastocysts

Surplus human blastocysts from clinical *in vitro* fertilization (IVF) treatment were donated after informed consent and approval of the local ethics committees at Göteborg University and Uppsala University. Donated embryos were cultured to blastocysts until the age of 6-7 days. Blastocysts were graded as described in PCT/EP02/14895. Spontaneously hatched blastocyst (line SA002 [B]) were placed directly in HES™ medium (Vitrolife AB, Sweden) supplemented with 4 ng/ml human recombinant basic FGF (Invitrogen), and 125 µg/ml hyaluronic acid (HA) (Ophthalin, CIBA Vision Nordic AB, Sweden) on a layer of mitotically inactivated early passage mouse embryonic fibroblasts (MEFs) (F1 hybrid CD1xC57BL/6, Charles River Laboratories, Sulzfeld, Germany). The MEF-cells were derived and cultured as previously described. Briefly, the mitotic activity of the MEFs was abolished by an incubation with 10 µg/ml Mitomycin C (Sigma-Aldrich, Sweden AB, Stockholm, Sweden) for three hours at 37° C, after which the cells were seeded at a density of 130,000 cells/ml in IVF cell culture dishes (Falcon 3653, Becton Dickinson, Franklin Lakes, NJ) in MEF-medium (D-MEM supplemented with 10 % fetal calf serum (FCS), 100 U/ml Penicillin G, and 1x Glutamax (Invitrogen, Sigma-Aldrich). Prior to the addition of treated blastocysts or hBS cells the MEF-medium was changed to HES™-medium. After plating the blastocysts on the MEF cells, growth was monitored and when the colony was large enough for manual passaging approximately 1-2 weeks after plating) the inner cell mass cells were dissected from other cell types and expanded by growth on new MEF cells.

Example 2

Establishment of hBS cell line from blastocysts with an intact zona pellucida

Blastocysts with intact zona were graded as described in PCT/EP02/14895 and randomly selected for either pronase treatment (lines Fertilitetscentrum (FC)018, Akademiska sjukhuset AS034, and AS038) or pronase treatment followed by immunosurgery (lines Sahlgrenska SA121, SA181) (see below).

The blastocysts were treated in pronase for 1-3 minutes (Sigma-Aldrich:10 U/ml in ICM-2, Vitrolife AB, 1-3 minutes in three subsequent drops), washed three times in ICM-2 and plated on MEFs in HES™-medium supplemented with 125 μg/ml HA and 4 ng/ml hrbFGF. ICM-2 is a blastocyst culture medium containing recombinant human albumin and HA. Pronase treated zona-free blastocysts selected for immunosurgery were washed twice in anti-human serum antibody (Sigma-Aldrich, 1:5 in ICM-2). After the second wash the blastocysts were placed in a new drop of the antibody and incubated for 30 minutes. The blastocysts were then extensively washed three times in ICM-2 medium followed by three washes in guinea-pig complement serum (Invitrogen, 1:5 in ICM-2). The blastocysts were then incubated for 10 minutes in guinea-pig complement serum and followed by three washes in ICM-2 and placed on MEFs in HES™-medium supplemented with 125 μg/ml HA and 4 ng/ml bFGF.

Example 3

Culturing of hBS cell line

ICM outgrowths were passaged to plates with fresh medium and MEF cells by mechanical dissection using Stem Cell Tool™ (Swemed Lab International AB, Billdal, Sweden). Established hBS cell lines were routinely passaged every 4-5 days. The hBS cell colonies were mechanically cut into pieces, 200 x 200 μm, and removed from the culture dish and transferred to a new culture dish with fresh MEF cells and hBS cell culture medium.

Example 4

Preparation of concentrated conditioned medium (CC base)

Approximately 10 hBS cell colonies were cultured with MEF cells (150.000 cells were seeded per dish) using a FCS based medium (15% Fetal Calf Serum, 1% v/v PEST,

1% v/v Glutamax, 1% v/v NEAA, 3.5 mM glucose and 4 ng/ml bFGF in KO-DMEM). The medium was collected every second day and concentrated in Centriprep concentration columns WM50 at 1500 g. 50 ml conditioned medium was concentrated 4 x (to 12.5 ml) to obtain the concentrated conditioned medium (CC base), aliquoted and frozen. Before use in cloning experiments, the aliquot was diluted 1:50 and sterile filtered (see Figure 1).

Example 5

Subcloning of hBS stem cell lines.

Preparation of embryonic mouse feeder layer (MFL)

200 µl MitC (Sigma) were added to 20 ml EMFI (500 ml DMEM/Glutamax, 50 ml FCS and 5.5 ml PEST). The media from the MEF cells that were thawed 4 to 5 days earlier was changed to the MitC containing medium and the cells incubated at 37°C and 5% CO2 for 2.5-3 hours. Preceding plating, MEF cells were washed 3 times in PBS (Invitrogen), dissociated by Trypsin/EDTA solution (Invitrogen) and plated in final concentrations from 150.000 cells/ml to 10.000 cells/ml in IVF-dishes, in 96-, 48, and/or 24-well-plates. Prior plating, the wells and dishes were coated with gelatine 2-3 hours (1 g gelatine dissolved in 1 l ddH2O and autoclaved).

Subcloning of hBs stem cell line

All the media was pre-warmed before use. HBS cells were washed inside the wells twice with cloning medium, 150 µl, 500 µl, and 1000 µl for the 96-, 48-, 24-well-plates, respectively. The inner part of the colonies was cut with a 300 µm Stem Cell Tool™ (Swemed Lab International AB) and subsequently incubated with 0.5 mM EDTA in PBS w/o Ca/Mg for 20 minutes at 37° C. The cells were triturated carefully with a pipette and diluted either in 1) a hBS cell conditioned cloning medium, (KO-DMEM medium supplemented with 15 % of concentrated conditioned medium, 3.5 mM glucose, 1 % Glutamax (Invitrogen), 1% PEST (Invitrogen), 1% NEAA (Invitrogen), and 4ng/ml bFGF (Sigma)), 2) a KO-DMEM-medium supplemented with 15% FCS, 3.5 mM glucose, 1% Glutamax, 1% PEST, 1% NEAA, and 4ng/ml bFGF, or 3) a KO-DMEM-medium supplemented with 20% serum replacement (Invitrogen) SR, 1% PEST, 1% Glutamax, 1% NEAA and 4ng/ml bFGF with or without 3.5 mM glucose. Single cells were picked and put into individual wells with MEF coated plates as well as wells without feeder cells. To confirm the colony forming ability of the cells, positive controls were performed (e.g. 100 and 1000 cells and smaller clusters were seeded in individual wells) as well

as negative controls (wells without dissociated hBS cells). After 24 hours 50 % v/v of the medium was changed to fresh hBS culture medium (composition mentioned above) in order to decrease the differentiation rate of the colonies. Further medium changes were performed twice a week and the plates were regularly screened for clones.

Colonies were cut with sharp capillaries and transferred as whole colonies or as clusters to feeder coated IVF dishes or dishes and plates without feeder cells, using Stem Cell Tool™. The following culture and expansion were performed according to standard protocols in order to generate sufficient material for characterization and vitrification.

Example 6
Immunocytochemistry

In order to characterize the subclones, the cells were fixed in 4 % PFA for 15 min at RT and exposed to the primary antibodies overnight at 4°C. As secondary antibodies (1:50 FITC –or Cy-3-conjugated antibodies (Southern Biotech) were used.

Primary antibodies against the cell surface antigens SSEA-1, SSEA-3, and SSEA-4 (stage specific embryonic antibodies) as well as the TRA1-60 and TRA1-81 were used to stain undifferentiated hBS cells.

The monoclonal antibodies (mAb) directed against SSEA-1, SSEA-3 and SSEA-4 (Developmental Studies Hybridoma Bank) were used all 1/200. TRA-1-60 and TRA-1-81 (Santa Cruz, Biotechnologies Inc) were used 1/100, neuroectodermal precursor cells were detected with a monoclonal antibody against nestin (BD Transduction Laboratories). Mesodermal cells were detected using a monoclonal antibody against desmin (Chemicon, International) and for endodermal cells the rbAb against HNF3b (Santa Cruz; 1/500) was used. Incubation in primary antisera was performed at 4°C overnight. Some cultures were double-stained with DAPI (4'-6'Diamidino-2-phenylindole, Sigma, final concentration 0.1 µg/ml, incubation for 10 minutes).

Example 7
Teratoma formation in immunodeficient mice

Severe combined immunodeficient (SCID)-mice, (Bosma and Carroll, 1991)(C.B-17/IcrCrl-scidBR, Charles River Laboratories, Sulzfeld, Germany) were used as animals host for the xenografted hBS cells. Four to five weeks old animals were anesthetized with intra peritoneal (i.p.) injections of ketamine hydrochloride (Ketalar, Warner Lambert Nordic AB, Solna, Sweden, 75 μg/g mouse) and medetomidine hydrochloride (Domitor, Orion Pharma Corporation, Espoo, Finland, 1 μg/g mouse). hBS cell colonies were mechanically cut into 200 x 200 μm pieces, washed once in Cryo-PBS (Vitrolife AB) and 20 cell clusters were injected under the kidney capsule or in the testicular lumen using a 300 μm lumen glass transfer pipette (Swemed Lab International AB). The number of cells transferred was approximately 20 000 to 40 000 per organ. Control animals were treated with Cryo-PBS injections and other control animals were grafted with primary brain cells from a littermate. The mice were resuscitated with i.p. injections of atipamezol (Antisedan, Orion Pharma, 1 μg/g mouse), and kept on a heated pad until consciousness. Palpable tumours started to appear three weeks after transplantation. The tumours were allowed to develop for eight weeks before the animals were sacrificed by cervical dislocation. All animals appeared healthy during the eight week period and no animals were deceased due to illness. The tumours were excised and immediately fixated in a 4 % solution of paraformaldehyde (PFA).

Results and discussion

In the inventors efforts to subclone cell line AS034, two clones AS034.1 and AS034.2 were obtained, showing a morphology comparable to cell line AS034 from which they were derived. To promote cell survival, cell free hBS cell conditioned medium derived from hBS cells grown in presence of FCS as cloning medium and FCS based medium were used (see Material and Methods). The overall yield was low; on average from approximately 10^3 dissociated single cells one colony resulted. However, both the hBS cell conditioned cloning medium and the FCS based cloning medium gave better results than the use of a serum replacement medium (see Figure 4).

When trying to subclone cell line SA002 (a cell line having a higher growth rate) a cloning efficiency >5% was achieved in a few experiments in which approximately 150 single cells were seeded to individual wells.

Characterization of subclone AS034.1 and AS034.2 revealed that they behaved comparable to the other cell lines in terms of the expression of the cell surface markers SSEA-4, SSEA-3, TRA-1-60 and TRA-1-81. Figure 5 shows, a colony of AS034.1 positive for the undifferentiated marker SSEA-4. Importantly, SSEA-1 and nestin was not detected in undifferentiated colonies. Furthermore, the subclones possess high levels of alkaline phosphatase (AP) activity, which is normally associated with undifferentiated colonies.

Furthermore, the subclones ware capable of differentiating into ectodermal, mesodermal, and endodermal cell types both *in vitro* and *in vivo*. The colonies were kept on mouse feeder layers for more than 7 days without passaging. However, already when grown to confluence and allowed to pile up in the culture dish, the subclones AS034.1 and AS034.2 differentiated spontaneously, even in the presence of mouse feeder layer cells and human bFGF. Without passing embryoid body stage colonies differentiate spontaneously into a variety of cell types, including all derivatives of the three embryonic germ layers ectoderm, mesoderm and endoderm. Indeed both lines are able to generate *in vitro* cells of the three germ layers and thus show no differences to cell line AS034 or other previously established hBSC lines (AS034.1 cultured without passaging for 12 days positive for the endoderamal marker HNF3β can be seen in Figure 6). As mentioned above both AS034.1 and AS034.2 express markers that are essential criteria of undifferentiated human blastocyst-derived stem cell lines. Therefore, the data suggest the pluripotency of the subclones AS034.1 and AS034.2. The teratomas derived from subclone AS034.1 showed a similar structure as those of the motherline AS034. All three germ layers were detected by microscopy analyzis of tumor sections, which also suggests the pluripotency of the clone (see Figure 7-9).

By definition, clonal expansion of hBS cells is a prerequisite for the strict definition of pluripotent cell line. The currently available culture conditions for clonal expansion of hBS cells are suboptimal. Unlike mouse BS cells, hBS cells die at high rate when they are dissociated into single cells. Depending on which cell line used, only 0.1-5% of plated single cells may be able to generate colonies that could be propagated. Among the few clones that survived the majority are lost due to irreversible differentiation. The present inventors found that concentrated conditioned medium from hBS cells grown in presence of serum, such as, e.g. FCS and optionally, subsequent change to a serum

free culture medium after a suitable time interval, promoted cell survival and maintenance of an undifferentiated fate.

Furthermore, culture conditions that may be rate-limiting for maintaining undifferentiated growth of hBS cells, include MEF quality and density, changes in the osmolality, pH, and temperature of the medium, as well as the presence of supplements, such as β-mercaptoethanol.

References

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2. Thomson, J.A. et al. Clonally derived human embryonic Stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture, Developmental Biology 227, 271-278 (2000)

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CLAIMS

1. A method for clonal derivation of human blastocyst-derived stem cells (hBS) or derivatives thereof, the method comprising the steps of

a) dissociation of hBS cell colonies into one or more single hBS cells,

b) cultivation of the one or more single hBS cells,

to obtain one or more hBS cell clones which are derived from a single hBS cell and are capable of forming colonies.

2. A method according to claim 1, wherein the cultivation in step b) is performed in a medium that promotes propagation of the one or more hBS cells.

3. A method according to claim 1 or 2, wherein the cultivation in step b) is performed in a cell free hBS cell conditioned cloning medium (CC medium).

4. A method according to claim 3, wherein the cell free hBS cell conditioned cloning medium comprises at least 5% v/v, such as, e.g., at least 7.5% v/v, at least 10% v/v, at least 12.5 % v/v, at least 15% v/v, at least 17.5% v/v, at least 20% v/v, at least 25% v/v, at least 30% v/v, at least 35% v/v, at least 40% v/v, at least 45 % v/v, at least 50% v/v, at least 55% v/v, at least 60% v/v, at least 65% v/v, at least 70% v/v, at least 75% v/v, at least 80% v/v, at least 85% v/v, at least 90% v/v, at least 95% v/v or 100% v/v of a concentrated conditioned medium (CC-base) and, optionally, a suitable cultivation medium.

5. A method according to claim 4, wherein the concentrated conditioned medium is prepared by the following steps:

1) cultivating hBS cells in a serum based medium, such as, e.g., FCS (Fetal Calf Serum) based medium or human serum based medium to obtain a conditioned medium,

2) collecting the conditioned medium within suitable time intervals,

3) concentrating the conditioned medium,

to obtain the concentrated conditioned medium (CC base).

6. A method according to claim 5, wherein step 3) is performed by concentrating the conditioned medium by a factor from about 2 to about 10, such as, e.g. from about 2 to about 9, from about 2 to about 8, from about 3 to about 7, from about 4 to about 6 or

from about 4 to about 5 by use of a suitable concentrating column.

7. A method according to claim 5 or 6, wherein the serum based medium is FCS based medium and comprises from about 60% v/v to about 90% v/v KO-DMEM medium, from about 5% v/v to about 30% v/v FCS, from about 1 mM to about 10 mM glucose and from about 1 ng/ml to about 20 ng/ml bFGF.

8. A method according to claim 7, wherein the FCS based medium further comprises from about 0.1% to about 5% PEST, from about 0.1% to about 5% v/v Glutamax and/or from about 0.1% v/v to about 5% v/v NEAA.

9. A method according to claim 8, wherein the FCS based medium comprises 15% FCS, 3.5 mM glucose, 1% PEST, 1% NEAA and 4 ng/ml bFGF in KO-DMEM.

10. A method according to any of claims 5-9, wherein the medium is collected at least every 12th hour, such as, e.g. at least every 18th hour, at least every 24th hour, at least every 36th hour, at least every 48th hour or at least every 60th hour.

11. A method according to any of claims 4-10, wherein the cultivation medium in the cell free hBS cell conditioned cloning medium (CC medium) is KO-DMEM-medium.

12. A method according to claim 11, wherein the cell free hBS cell conditioned cloning medium further comprises glucose.

13. A method according to claim 11 or 12, wherein the cell free hBS cell conditioned cloning medium (CC medium) further comprises Glutamax, NEAA, PEST and/or bFGF.

14. A method according to claim 13, wherein the cell free hBS cell conditioned cloning medium comprises 81% KO-DMEM, 15% concentrated conditioned medium (CC base), 3.5 mM D-glucose, 4 ng/ml bFGF, 1% v/v PEST, 1% v/v Glutamax and 1% v/v NEAA.

15. A method according to claim 1 or 2, wherein the cultivation in step b) is performed in a Fetal Calf Serum (FCS) based medium comprising at least 5% v/v FCS, such as, e.g., at least 7.5% v/v FCS, at least 10% v/v FCS, at least 12.5 % v/v FCS, at least 15% v/v FCS, at least 17.5% v/v FCS, at least 20% v/v FCS, at least 25% v/v FCS, at

least 30% v/v FCS, at least 35% v/v FCS, at least 40% v/v FCS or at least 45 % v/v FCS and a suitable cultivation medium.

16. A method according to claim 15, wherein the cultivation medium comprises KO-DMEM medium.

17. A method according to claim 15 or 16, further comprising a growth factor, such as, e.g. bFGF, EGF, HGF and/or FGF4.

18. A method according to any of claims 15-17, further comprising glucose.

19. A method according to claim 18, wherein the FCS based medium comprises from about 60% v/v to about 90% v/v KO-DMEM medium, from about 5% v/v to about 30% v/v FCS, from about 1 mM to about 10 mM glucose and from about 1 ng/ml to about 20 ng/ml.

20. A method according to any of claims 15-19, wherein the FCS based medium further comprises Glutamax, NEAA and/or PEST.

21. A method according to claim 1 or 2, wherein the cultivation in step b) is performed in a Human Serum based medium comprising Human Serum and a suitable cultivation medium.

22. A method according to any of the preceding claims, wherein step b) is performed in the presence of embryonic fibroblasts, such as, e.g. mouse embryonic fibroblasts, human foreskin fibroblasts, fetal skin fibroblasts, fetal muscle fibroblasts, adult skin fibroblasts and fibroblasts derived from hBS cells.

23. A method according to any of claims 1-21, wherein step b) is performed under feeder cell free conditions.

24. A method according to claim 23, wherein step b) is performed on a support medium comprising a component that promotes colony formation and/or cell division and/or adhesion and/or inhibits differentiation of the hBS single cells, such as, e.g. albumin, gelatine, poly-ornithine, fibronectin, vitronectin, agarose, poly-L-lysine, collagen, and/or extracellular matrix components, such as, e.g. Matrigel® or laminin and/or combinations

thereof.

25. A method according to any of the preceding claims, wherein the hBS cell colonies in step a) are dissociated without the use of enzymatic treatment.

26. A method according to claim 25, wherein the hBS cell colonies in step a) are dissociated by use of a mechanical method.

27. A method according to claim 26, wherein the mechanical method comprises the steps of

i) cutting hBS cell colonies to obtain smaller units.

ii) incubating the smaller units with a medium containing a chelator such as, e.g., EDTA,

iii) triturating the smaller units to obtain hBS single cells.

28. A method according to claim 27, further comprising a step of

iv) dispersing the hBS single cells in a suitable medium, such as, e.g. a cell free hBS cell conditioned cloning medium (CC-medium) or FCS based medium.

30. A method for preparing a concentrated conditioned medium (CC base), the method comprising

1) cultivating hBS cells in a serum based medium, such as, e.g., FCS (Fetal Calf Serum) based medium or human serum based medium,
2) collecting the conditioned medium within suitable time intervals,
3) concentrating the conditioned medium,

to obtain the concentrated conditioned medium (CC base).

31. A method according to claim 30, wherein step 3) is performed by concentrating the conditioned medium by a factor from about 2 to about 10, such as, e.g. from about 2 to about 9, from about 2 to about 8, from about 3 to about 7, from about 4 to about 6 or from about 4 to about 5 by use of a suitable concentrating column.

32. A method according to claim 30 or 31, wherein the serum based medium is a FCS based medium and comprises from about 60% v/v to about 90% v/v KO-DMEM medium, from about 5% v/v to about 30% v/v FCS, from about 1 mM to about 10 mM glucose and from about 1 ng/ml to about 20 ng/ml bFGF.

33. A method according to claim 32, wherein the FCS based medium further comprises from about 0.1% to about 5% PEST, from about 0.1% to about 5% v/v Glutamax and/or from about 0.1% v/v to about 5% v/v NEAA.

34. A method according to claim 33, wherein the FCS based medium comprises 15% FCS, 3.5 mM glucose, 1% PEST, 1% NEAA and 4 ng/ml bFGF in KO-DMEM.

35. A method according to any of claims 30-34, wherein the medium is collected at least every 12th hour, such as, e.g. at least every 18th hour, at least every 24th hour, at least every 36th hour, at least every 48th hour or at least every 60th hour.

36. A method according to any of claims 30-35, wherein the cultivation medium in the cell free hBS cell conditioned cloning medium (CC medium) is KO-DMEM-medium.

37. A method according to claim 36, wherein the cell free hBS cell conditioned cloning medium further comprises glucose.

38. A method according to claim 36 or 37, wherein the cell free hBS cell conditioned cloning medium (CC medium) further comprises Glutamax, NEAA, PEST and/or bFGF.

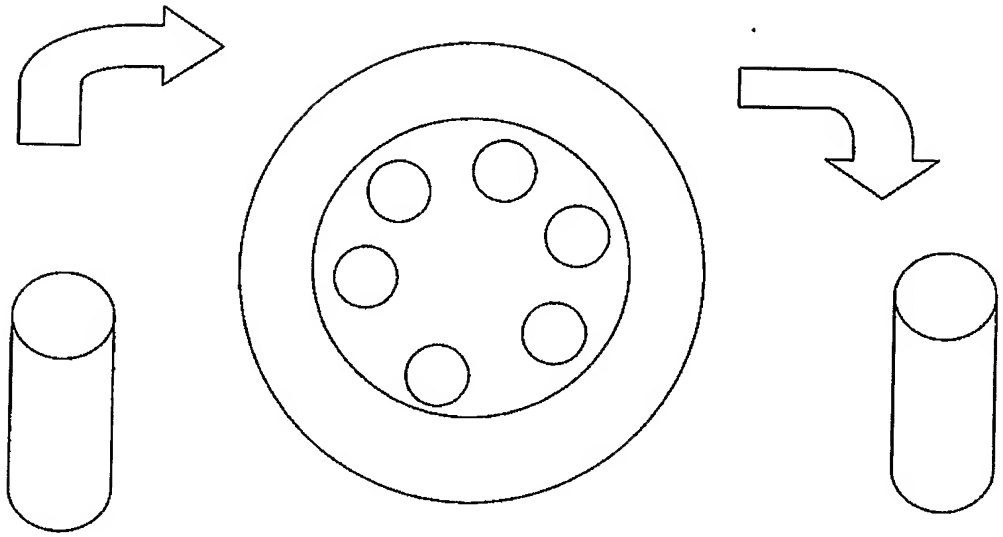
39. A method according to claim 38, wherein the cell free hBS cell conditioned cloning medium comprises 81% KO-DMEM, 15% concentrated conditioned medium (CC base), 3.5 mM D-glucose, 4 ng/ml bFGF, 1% v/v PEST, 1% v/v Glutamax and 1% v/v NEAA.

40. A hBS cell conditioned cloning medium comprising a concentrated conditioned medium prepared by a method as described in any of claims 30 – 39.

41. A kit for performing the method described in any of claims 1-28, comprising at least two of the following components in separate compartments: a cell free hBS cell conditioned cloning medium (CC medium), a concentrated conditioned medium (CC base), HBS medium without beta-mercaptoethanol and human or mouse embryonic feeder cells.

42. A kit for performing the method described in any of claims 1-28, comprising at least two of the following components in separate compartments: a cell free hBS cell

conditioned cloning medium (CC medium), a concentrated conditioned medium (CC base), a hBS culture medium HES medium, and a suitable support medium comprising a component that promotes colony formation and/or cell division and/or adhesion and/or inhibits differentiation of the hBS single cells, such as, e.g. albumin, gelatine, poly-ornithine, fibronectin, agarose, poly-L-lysine, collagen, and/or extracellular matrix components, such as, e.g. Matrigel® or laminin and/or combinations thereof.



X 4

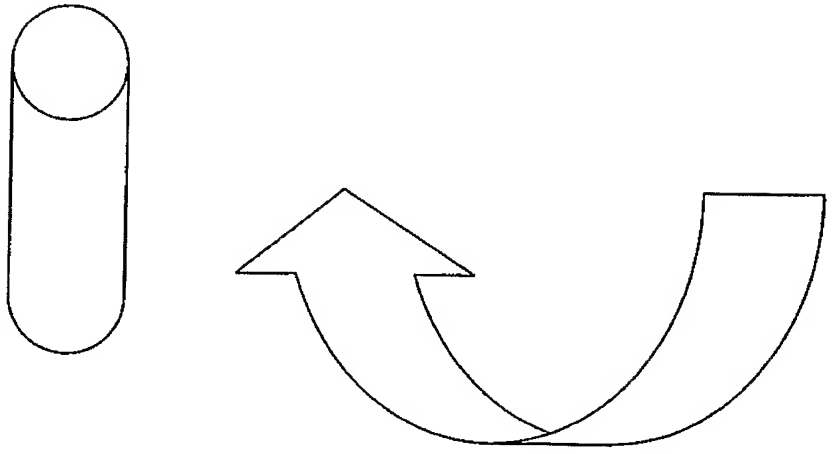


Figure 1

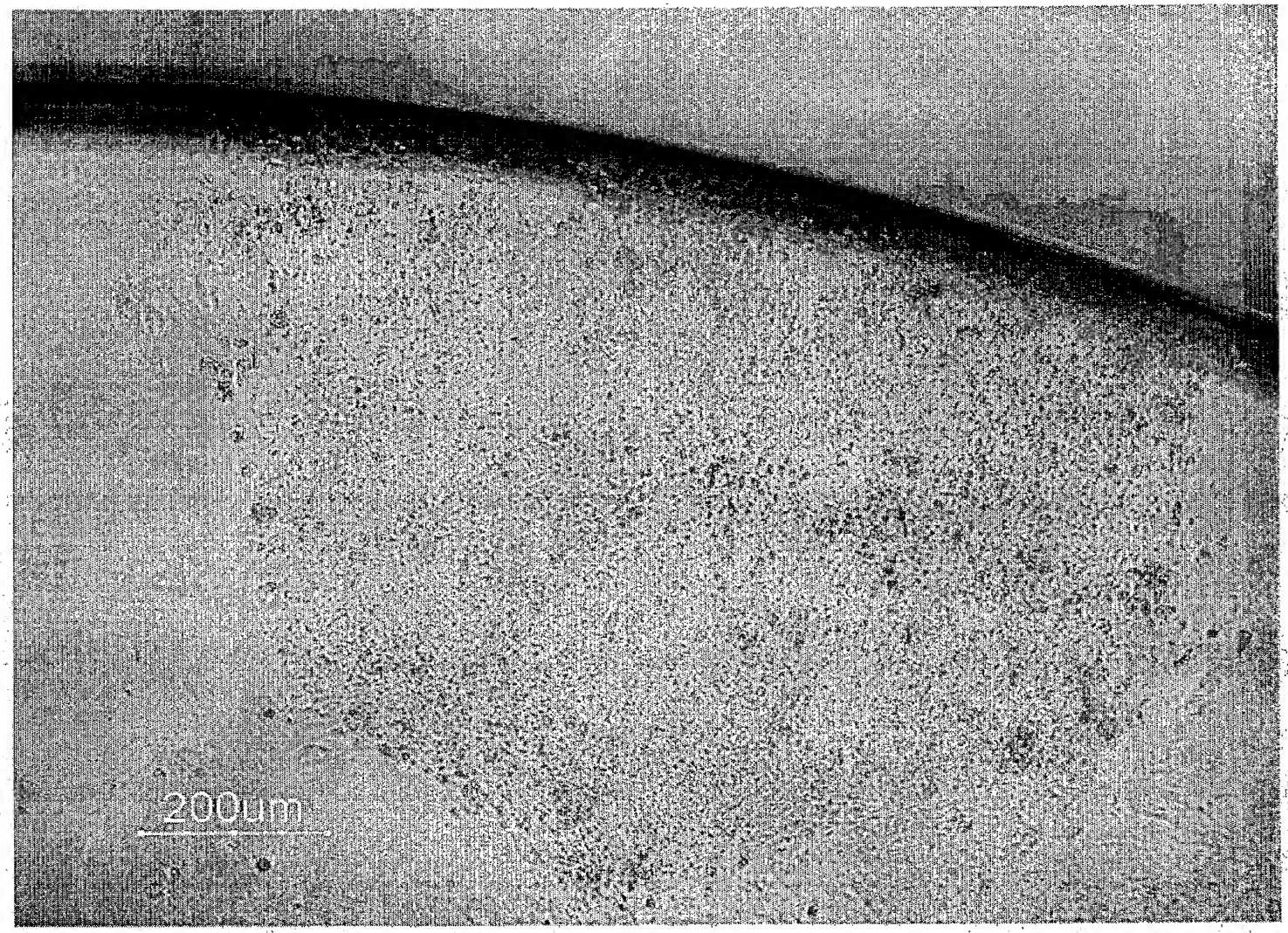


Figure 2

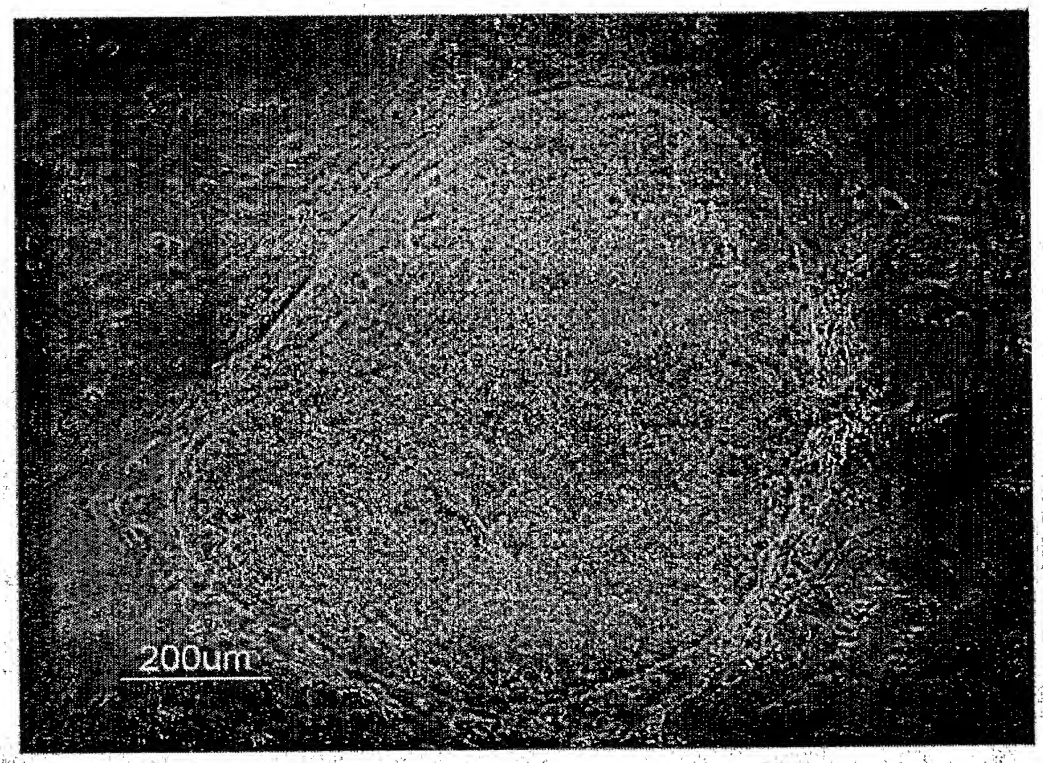


Figure 3

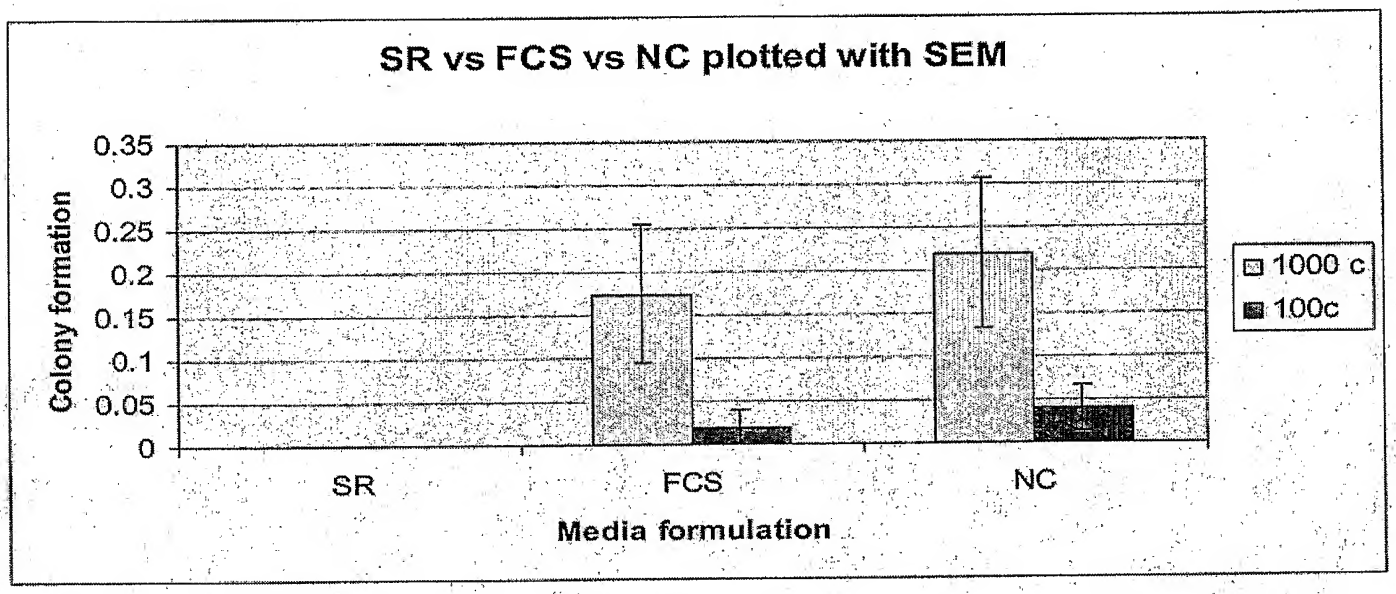


Figure 4

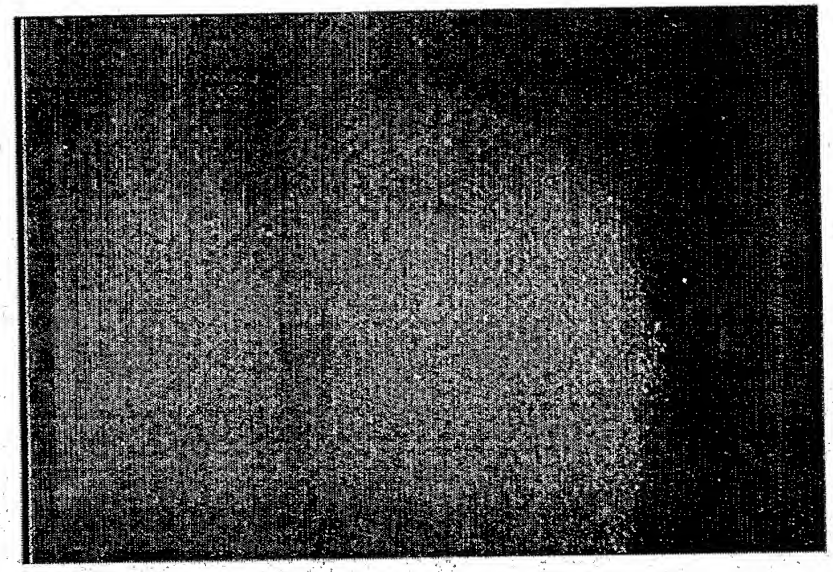


Figure 5

Figure 6

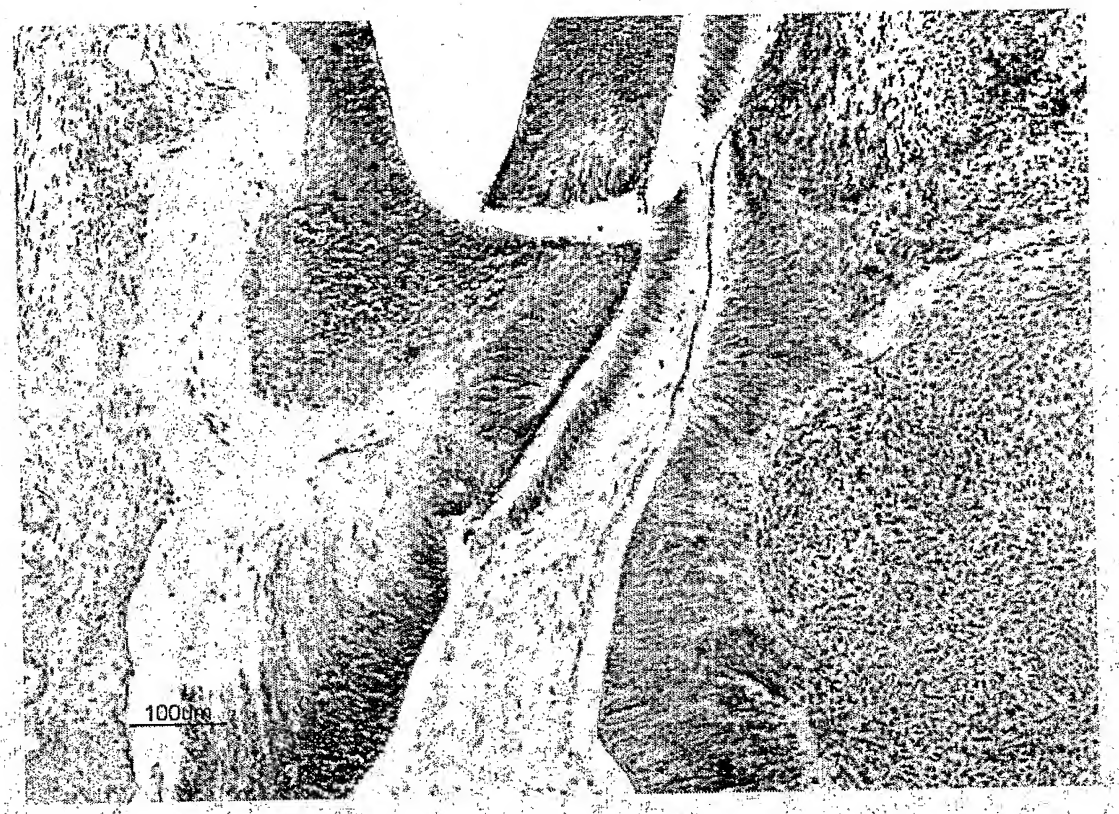


Figure 7

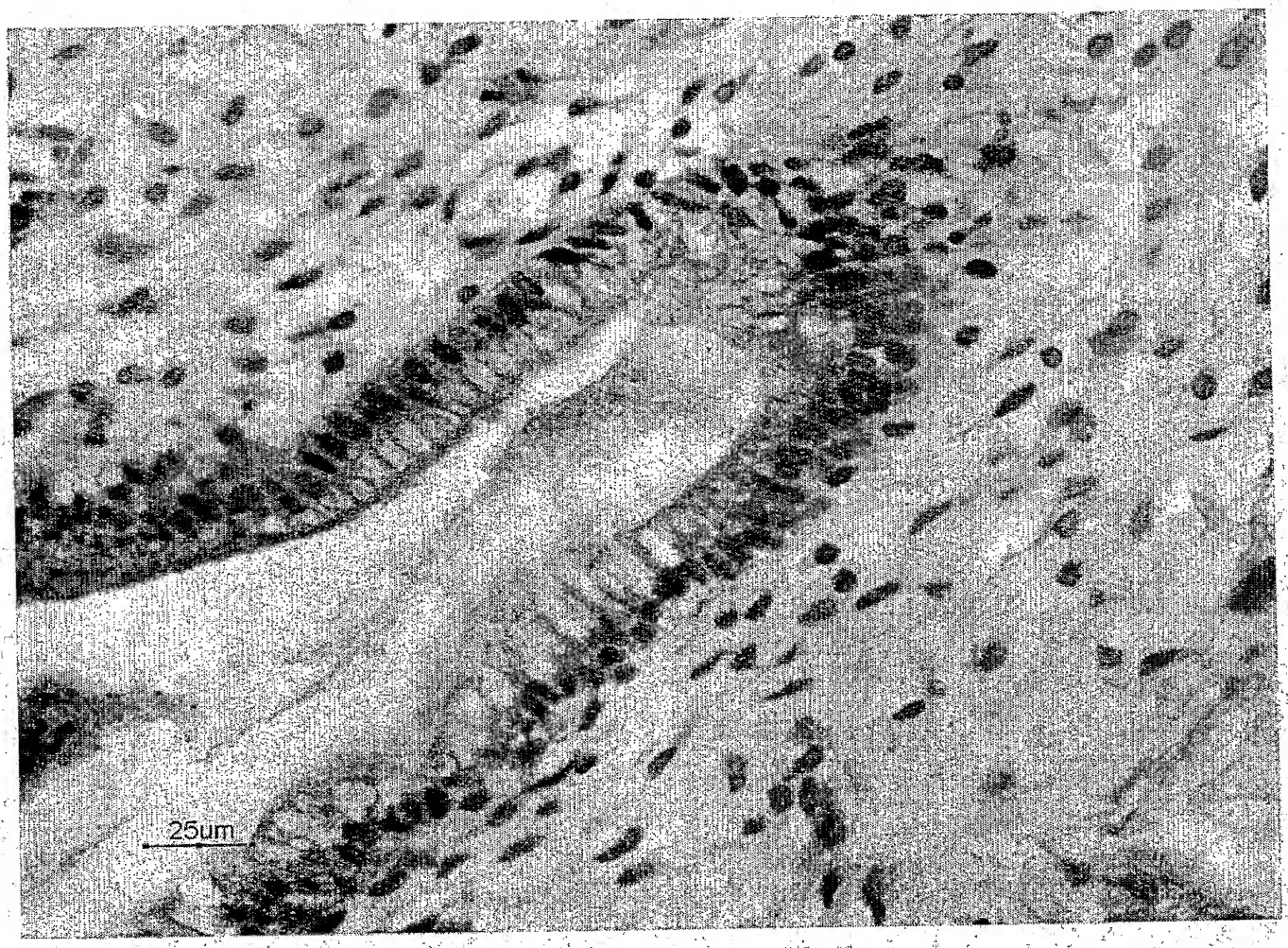


Figure 8

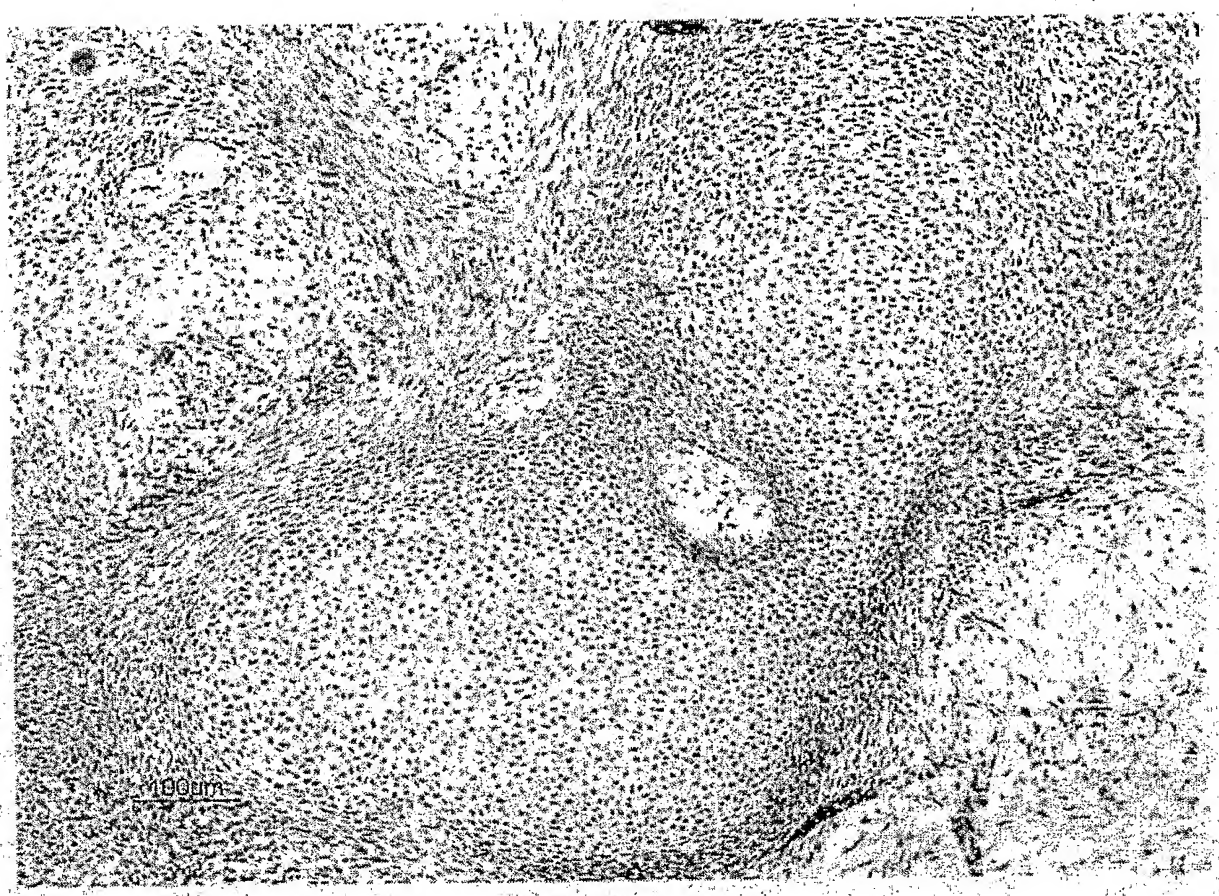


Figure 9